A CONTRIBUTION TO THE STUDY OF THE PRECIPITINS.*

By H. G. Chapman, M.D., B.S.

(From the Physiological Laboratory of the University of Sydney.)

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General historical introduction.

The existence of specific precipitins in the blood was first demonstrated in 1897 by Kraus,† who added cholera, plague, and typhoid antisera to filtered cultures of the corresponding germs. On the addition of the homologous culture-filtrate a precipitate was formed, but this did not appear if the antiserum was not homologous. These observations were confirmed by Nicolle,‡ who employed cultures of *Bacillus coli*, *B. typhi*, and *Vibrio massuali*. These investigators called the bodies present in the antisera employed by them agglutinins. In 1899 Bordet§ noted that rabbits, which had received several intraperitoneal

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† Wien. klin. Wochenschr. x , S.736, 1897, quoted from Nuttall, Journ. of Hygiene, i., p.368, 1901.

‡ Ann. de l'Inst. Pasteur, xii., p.161, 1898.

§ Ann. de l'Inst. Pasteur, xiii., p.232, 1899.

injections of defibrinated blood from fowls, yielded a serum not only possessed of hæmolytic power against the red corpuscles of the fowl but also capable of giving rise, when mixed with fowl's serum, to a precipitate which slowly became abundant and aggregated into flocculi. In the same paper Bordet described "lactosera." He applied this term to antisera which precipitated caseinogen. Bordet* stated that this property of antisera had already been investigated by Tchistovitch. Tchistovitch† stated that, on mixing the serum of the eel with a strong antitoxin from an animal (rabbit, hare, guinea-pig or dog) which had been immunised for a little time, a cloud and a precipitate similar to that which had been observed by M. Kraus in filtered cultures of germs, were obtained. This precipitate was insoluble in water, neutral salts and alkaline carbonates, but dissolved easily in alkalies and acids. Its formation resembled the coagulation of a substance dissolved in the toxic serum or antitoxin.

In 1900 Uhlenhuth‡ commenced his researches on the precipitins with a contribution on specific tests for egg-albumens. He concluded that, by repeated intraperitoneal administration of a solution of hen's egg-white into rabbits, there were formed in the serum bodies which, on their addition to solutions of hen's egg-white, gave rise to a cloud or precipitate; that similar results were seen with solutions of pigeon's egg-white; that the serum of rabbits treated with solutions of pigeon's egg-white contained bodies which produce clouds and precipitates in solutions of the egg-white of the fowl and pigeon; that the reaction so produced occurred only with egg-white, not with the numerous other proteins tested, and that the reaction showed great delicacy. In a later paper§ Uhlenhuth concluded that it would not be possible to differentiate eggs as had been possible for bloods. Gengou‖ found that he was unable to observe any difference in the action of hen's

* Ann. de l'Inst. Pasteur, xiii., p.285, 1899.
† Ann. de l'Inst. Pasteur, xiii., p.406, 1899.
‡ Deutsch. med. Wochenschr. xxvi., S.734, 1900.
§ *Ibid.* xxvii., S.260, 1901.
‖ Ann. de l'Inst. Pasteur, xvi., p.734, 1902.

egg-white antiserum upon solutions of the egg-whites of the fowl, duck, pigeon, and turkey. Nuttall,* Levene,† and Graham-Smith‡ obtained analogous results. In 1901 Uhlenhuth§ applied this biological method to the separation of different kinds of blood, and especially to the identification of human blood. Wassermann and Schutze,‖ Mertens,¶ Stern,** Nuttall and Dinkelspiel,†† Zuelzer‡‡ and Ziemke§§ made important investigations on these applications of the test.

Kowarski|‖ immunised rabbits with extracts of wheaten meal from which the protein coagulated by heat had been filtered off The antisera from such rabbits gave a precipitate with the extract used for immunisation. Similar precipitates were given with extracts of rye and barley. No precipitate occurred with extract of oats. A pronounced cloud formed with extract of peas. It was concluded that the vegetable proteins were not of such different kinds as the animal proteins

Bertarelli¶¶ examined the relations of the proteins of the bean, pea, lentil, and vetch. He injected dogs with the extracts from these seeds, and obtained weak antisera. No qualitative specificity could be detected.

Relander*** prepared antisera for the vetch and barley. He found the antisera to be specific for the two substances tested.

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* Journ. of Tropical Med., iv., p.408, 1902.

† Medical News, lxxix., p.981, 1901, quoted by Nuttall, Blood Immunity and Relationship, Cambridge, 1904.

‡ Nuttall, Blood Immunity and Relationship, Cambridge, 1904.

§ Deutsch. med. Wochenschr., xxvii., S.83, 499, and 780, 1901.

‖ Deutsch. med. Wochenschr., xxvii., Vereinbeilage, S.80, 1901.

¶ Deutsch. med. Wochenschr.. xxvii., S.161, 1901.

** Deutsch. med. Wochenschr., xxvii., S.135, 1901.

†† Journ. of Hygiene, i., p.367, 1901.

‡‡ Deutsch. med. Wochenschr., S.219, 1901.

§§ Deutsch. med. Wochenschr., xxvii., S.424 and 731, 1901.

|‖ Deutsch. med. Wochenschr., xxvii., S. 442, 1901.

¶¶ Cent. f. Bact. xi., S.8, 1903.

*** Cent. f. Bact. xx., S.518, 1908.

Precipitin antisera have also been prepared for ricin,* crotin, and ergot.† These have all shown a high degree of specificity.

Scope of Inquiry.

The study of the precipitins, as these bodies came to be called, progressed rapidly in the next few years, since they seemed to be concerned with those specific properties of the components of the tissues of different genera and species which had evaded the chemical tests formerly employed. Some investigators turned their attention to the details of the use of the test for the recognition of the blood and proteins of different forms in cases of legal interest. Others endeavoured to see whether the relations revealed in the zoological kingdom by the biological method were similar to those determined on morphological homologies. Others, again, examined the conditions of formation of these substances in the living body in the hope that they might lead to further insight into those complex interactions by which the animal organism defends itself against noxious microbic and chemical agents. In this way certain definite paths of research became established. With some of these lines of research the experiments recorded in this paper are concerned. In the first place, a further attempt has been made to elucidate the interaction between antiserum and homologous protein with the object of eliciting the quantitative relationships of the reaction. In the second place, the knowledge of the numerical relations of antiserum and protein has been utilised to arrange methods for the differentiation of the proteins of closely allied species, and to render more accurate the diagnosis of the source of individual proteins. In the third place, an attempt has been made to determine by the biological method the relationship of proteins of vegetable origin. The greatly improved knowledge of the technique for the use of precipitins will now enable differences to be recognised in these proteins. And, lastly, the application of the results obtained by gravimetric means to the deviation of complement is pointed out.

* Jacoby, Hfm. Beitr. i., S.51, 1901.

† Ottoleughi, Biochem. Cent. 1902, No.1435.

General Remarks on the Methods employed.

The precipitin antisera were produced by the intraperitoneal injection of the serum, egg-white or solution containing protein into rabbits. In a few instances cats were employed. Subcutaneous and intravenous injections were attempted, but these methods of administration were not satisfactory. The rabbits were well fed, and frequently gained weight during immunisation. The large amount of food eaten was noticed. A rabbit weighing 1200 gm. would eat 400-600 gm. carrots in 24 hours. The rabbits were not fed on the day of injection until after the injection had been made. During the last three years the precaution of washing and shaving the rabbits' skins has been dispensed with. It was, however, of great importance that the syringe, needle, and fluid to be injected should be free from noxious organisms. The needle and syringe were rinsed in boiling water immediately before each injection. This simple but efficient method has much diminished the time of injecting the rabbits, so that twelve rabbits can be injected in a few minutes. Injections were at first made every 4 or 5 days, but experience soon showed that an injection every second day was well tolerated. In the last few immunisations daily injections were employed. The rabbits were weighed daily, and a sudden fall of weight was regarded as a sign to avoid injection for a day or two. The amount injected varied with the material used. Egg-white antisera seemed more powerful when the total amount of egg-white injected was large, so that 10 c.c. or 20 c.c. were given at each dose. With blood sera care had to be taken with the initial injections, which were usually 3 c.c. to 5 c.c. If the animal did not lose weight, the dose was increased to 10 cc. From six to eight injections were given. As very powerful antisera were obtained with five or six injections of small amounts of protein, making the total amount injected as 0·2 to 0·3 gm. dried protein equal to 2 to 3 c.c. blood serum, it was evident that the quantity of protein injected did not necessarily determine the amount of precipitin in the antiserum.

The animals were killed 7 to 12 days after the last injection. Nuttall's method* was followed. The blood was caught in a large sterile dish which was at once covered. The serum was removed from the dish in 7 or 8 hours, and either sealed up in sterile tubes and stored on ice, or dried† in vacuo over calcium-chloride at 37°C. Dried antisera were kept in a securely closed sterile bottle. Every precaution was taken to work under aseptic conditions. All the tubes used for the interactions, all pipettes, measuring cylinders, and other apparatus were sterilised in a Koch's steam steriliser. The salt solution used as the diluent was sterilised by raising to the boiling point on three successive days. The trouble of these precautions was amply repaid by the tubes remaining sterile for 7 or 8 days after being set up. All the tubes were plugged with sterile cotton wool. When a bacterial deposit may have simulated or concealed a slight precipitate, the tubes have been rejected.

Measurements have been made with pipettes graduated to 1/100th of a cubic centimetre, and corrected by weighing the quantity of mercury delivered by them.

The tubes employed for the interactions were made of ordinary glass tubing of about 5 mm. bore. The ends were drawn out and rounded off. The bottom of each tube was thus shaped as a cone. For certain experiments, tubes of a larger bore—8 mm.—were employed.

The Relations of the Interacting Substances.

The characters of the interaction between the antiserum and the protein employed for its production, have been studied to determine the general laws governing the reaction. If a given weight or volume of antiserum be allowed to interact with increasing quantities of protein, the precipitate formed increases

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* Nuttall, Blood Immunity and Relationship, Cambridge, 1904, p.59.

† On the advantage of using dried antisera, see Chapman, Proc. Linn. Soc. N. S. Wales, 1905, xxx., p.392.

up to a maximum. Under certain conditions, Arrhenius and Hamburger have found that a precipitate may diminish again.

Some qualitative experiments may be quoted first. A series of tubes were arranged containing from 0·05 to 0·000000005 gm. dried horse-serum dissolved in 0·5 c.c. saline solution (0·75 per cent. sodium chloride) so that the dilution of the dried horse-serum in 0·5 c.c. saline solution varied from 1 in 10 to 1 in 10,000,000. A control tube containing 0·5 c.c. saline solution alone was also prepared. To each tube of the first series and to the control was added 0·01 gm. dried antiserum (prepared in a rabbit by the injection of 4·4 gm. dried horse-serum in 8 doses) previously dissolved in 0·5 c.c. saline solution. The precipitates were read in 48 hours by measuring the length of tube occupied by the precipitate. A record of the readings is given in Table i.

Table i.

No. of tube.	Grams of dried horse-serum in each tube.	Dilutions of dried horse-serum in 0·5 c.c. saline.	Precipitates in tubes to which 0·01 gm. dried antiserum was added.
1	0·05	1 in 10	1 mm.
2	0·005	1 in 100	1·5 mm.
3	0·0005	1 in 1,000	1·5 mm.
4	0·00005	1 in 10,000	1·5 mm.
5	0·000005	1 in 100,000	0·5 mm.
6	0·0000005	1 in 1,000,000	0·3 mm.
7	0·00000005	1 in 10,000,000	0·3 mm.
8	control.	saline alone.	none.

From this Table it is evident that in tubes 1, 2, 3, and 4 maximal precipitates have been produced, while in tubes 5, 6 and 7 submaximal precipitates have been formed. After 48 hours the superfluids above the precipitates were removed and 0·2 c.c. from each tube was placed in a clean tube. To each of these tubes containing superfluid, 0·0001 gm. dried horse-serum dissolved in 0·5 c.c. saline was added. After 48 hours the readings of these secondary interactions were taken. They are recorded in Table ii.

These results* show that there was no additional precipitate in tubes 2 and 3, and small precipitates in tubes 4, 5, 6, 7 and 8.

Table ii.

No. of tube.	Precipitates after the addition of horse-serum.
1	not tested.
2	none.
3	none.
4	0·3 mm.
5	0·5 mm.
6	0·5 mm.
7	0·5 mm.
8	0·5 mm.

The maximum amount of precipitate is thus given by an amount of homologous protein which is adequate to precipitate all (or practically all) the precipitin. No further addition of protein will augment the precipitate.

Another series of secondary tubes was also prepared by placing 0·2 c.c. superfluid of each of the primary tubes in clean tubes. To each was added 0·01 gm. dried antiserum dissolved in 0·5 c.c. saline solution. These secondary tubes were read in 48 hours. The results are recorded in Table iii.

Table iii.

No. of tube.	Precipitates after the addition of antiserum.
1	not tested.
2	2·5 mm.
3	1·5 mm.
4	0·5 mm.
5	0·3 mm.
6	0·3 mm.
7	0·3 mm.
8	none.

* The reading of tube 1 is too small, since the interaction is not complete in 48 hours; compare Welsh and Chapman, Journ. of Hygiene, vi., p.258, 1906.

Precipitates were obtained in each tube. The homologous protein was thus not exhausted in any tube, and in spite of the fact that only one-fifth of the amount originally present was taken, it was still capable of eliciting from a second quantity of antiserum precipitates not less abundant than those obtained in the primary interactions. An apparent exception is tube 4, in which occurred a deposit much smaller than that primarily given. But the smaller precipitate in this instance reinforces the argument, since it is in all probability attributable not to the exhaustion of the homologous proteid but to the fact that the amount of horse-serum (0·00005 gm.) originally present was just insufficient to neutralise 0·01 gm. antiserum, and that the amount

Table iv.

No. of tube.	Amount of dried egg-white in grams.	Amount of dried antiserum in grams.	Precipitates in 48 hours.
1	0·0000012	0 01	0·3 mm.
2	0·0000024	,,	,,
3	0·0000036	,,	,,
4	0·0000048	,,	,,
5	0·0000060	,,	,,
6	0·0000072	,,	,,
7	0·0000084	,,	,,
8	0·0000096	,,	1 mm.
9	0·0000108	,,	,,
10	0·000012	,,	,,
11	0·000024	,,	,,
12	0·000036	,,	,,
13	0·000048	,,	,,
14	0·000060	,,	,,
15	0·00012	,,	,,
16	0·00060	,,	,,
17	0·00012	none.	none.
18	none.	0·01	none.

actually present in thesecondary reaction was 0·00001 gm.(already once acted on), making the interacting quantities more nearly equal to those in the primary tube 5. In tubes 4, 5, 6 and 7 uncombined precipitin (antiserum) coexisted with uncombined homologous protein in the clear superfluid.

A series of experiments illustrative of the action between fowl's egg-white and fowl's egg-antiserum may now be described. The rabbit was immunised by the injection of 6·48 gm. dried egg-white in six doses. The antiserum was dried. Egg-white was diluted with saline solution to facilitate measurement. The solid content of the egg-white was determined by drying in vacuo over calcium chloride to constant weight. All the primary tubes were made up to 2·6 c.c. with 0·75 % sodium chloride solution Table iv. records the primary interactions.

After 48 hours the superfluid above each precipitate was removed and filtered. The removed fluid was divided into three portions of 0·5 c.c. each, A, B, and C. To each tube of series A, 0·1 c.c. of 1 % egg-white in saline solution (0·00012) was added. To each tube of series B, 0·01 gm. dried antiserum dissolved in

Table v.

No. of tube.	Precipitates in series A (plus egg-white).	Precipitates in series B (plus antiserum).	Precipitates in series C (control).
1	1 mm.	0·3 mm.	none.
2	,,	,,	,,
3	,,	,,	,,
4	,,	0·5 mm.	,,
5	,,	,,	,,
6	0·5 mm.	,,	,,
7	,,	,,	,,
8	,,	,,	,,
9	,,	1 mm.	,,
10	0·3 mm.	,,	,,
11	trace.	,,	,,
12	,,	,,	,,
13	none.	2 mm.	,,
14	,,	,,	,,
15	,,	,,	,,
16	,,	,,	,,
17	,,	,,	,,
18	1 mm.	none.	,,

0·1 c.c. saline solution was added. The tubes of series C formed controls. After 48 hours, readings were taken, and these are recorded in Table v.

The results of this experiment show that in reactions 1 to 12 a quantity of precipitin has not been neutralised by the albumen. In reactions 13 to 16 the precipitin has been neutralised. It also shows that in every tube in which egg-white was present the further addition of antiserum caused a precipitate. On examination of the quantities it will be seen that a quantity of egg-white between 0·000036 gm. and 0·000048 gm. completely neutralised the precipitin in 0·01 gm. antiserum. The deposits given in Table v., Series A, show that the amount of precipitin decreased in tube 1 to tube 12, and that very little was present in tubes 11 and 12. It should be noted that not more than one-fifth of the original precipitin can be present in the tubes of Series A, and one-fifth of the original egg-white in the tubes of Series B recorded in Table v. The results of these typical experiments have been confirmed by comparison with similar experiments. Thus five other series have been made between solutions of fresh and dried egg-white and five other fowl's egg-white antisera (two of which were not dried), one series between ostrich egg-white and dried ostrich egg-white antiserum, one series between the serum of the dasyure and an antiserum for dasyure serum, one series between the serum of the bandicoot and an antiserum prepared against the serum of the bandicoot, and one series between fresh horse and a corresponding antiserum. From similar data Welsh and Chapman* concluded that, for constant quantities of precipitin interacting with varying amounts of homologous protein, the amount of precipitum remains constant as soon as sufficient homologous protein is present to neutralise all the precipitin.

These experiments serve to determine approximately the quantity of homologous protein which will neutralise the precipitin in a given weight of antiserum. Table vi. records the results obtained.

It is of interest to record in Table vii., the maximal precipitates obtained on complete neutralisation of the precipitin in these antisera.

* Journ. of Hygiene, vi., p.259, 1906.

These maximal precipitates were obtained by mixing 0·02 gm. dried antiserum and 0·004 gm. dried homologous protein. The reaction was allowed to take place in a pipette graduated to

Table vi.

Antiserum.	Weight in grams of protein always insufficient to neutralise 0·01 gm. antiserum.	Weight of protein usually sufficient to neutralise 0·01 gm. antiserum.	Weight in grams of protein always sufficient to neutralise 0·01 gm. antiserum.
Hen egg 1	0·000012	0·000024	0·000048
Hen egg 2	0·00001	0·00002	0·00003
Hen egg 3	0·00001	0·000025	0·00006
Ostrich egg	0·0005	—	0·002
Horse serum 1	0·00005	0·0001	0·0005
Horse serum 2	0·0005	—	0·002
Dasyure serum	0·0001	—	0·0005

hundredths of a cubic centimetre. The volume of the precipitate was read in 48 hours. On comparing Tables vi. and vii., it is seen that there is no correspondence between the amount of protein

Table vii.

Antiserum.	Volume of precipitum from 0·01 gm. dried antiserum.
Hen egg 1	0·024 c.c.
Hen egg 2	0·021 c.c.
Hen egg 3	0·010 c.c.
Ostrich egg	0·10 c.c.
Horse serum 1	0·024 c c.
Horse serum 2	0·008 c.c.
Dasyure serum	0·0025 c.c.

necessary to neutralise the precipitin in an antiserum and the amount of precipitate to be obtained from an antiserum on neutralising the precipitin completely.

Other series of experiments have been carried out in which the amount of homologous protein was constant and the amount of antiserum was varied. Such a series is recorded in Table viii.

It will be seen from the results recorded in the Table that the amount of precipitate is directly proportional to the amount of antiserum, *i.e.*, the quantity of precipitin.

Table viii.

No. of tube.	Weight of dried egg-white in gms.	Weight of dried antiserum in gms.	Precipitates after 48 hours in mm.
1	0·0001	0·01	2·0
2	0·0001	0·0075	1·5
3	0 0001	0·005	1·0
4	0·0001	0·0025	0·5
5	0 0001	0·001	trace.

More accurate methods, however, are required to determine the relation between the weights of interacting antiserum and homologous protein and the weight of the precipitate formed. The precipitates have been, therefore, weighed, and an examination of the precipitin reaction carried out by gravimetric methods. Measured quantities of fluid antiserum or weighed quantities of dried antiserum have been mixed with weighed amounts of protein in large tubes for the centrifuge. The tubes have been made up to a fixed volume with saline solution and allowed to stand 48 hours for the interaction to take place. The superfluid above the precipitate has been removed with a pipette and the precipitate washed five times with saline solution. Each time the precipitate has been mixed with 50 c.c. saline solution and the precipitate separated by spinning in the centrifuge. The precipitate has been then washed five times in the same way with 50 c.c. distilled water. The precipitate has been transferred to small glass tubes with thin walls, weighing about 4 gms. These tubes could be spun in a small centrifuge, and in this way the precipitate has been washed with absolute alcohol and finally with ether free from water. The tubes with their contents have been placed in an oven at 80°C. for several hours and thence put into the desiccator. The tubes have been kept a fixed time in the desiccator and weighed. The weights have been checked three

times. The tubes of a series have been weighed immediately after each other. Owing to the hygroscopic nature of the precipitates the error in the weights of the tubes has been found by experiment to be 0·3 mg. The mean figure of the several weighings has been taken in all cases.

Experiments were performed to ascertain the weight of precipitate obtained when a measured quantity of antiserum was allowed to interact with increasing weights of homologous protein. It has already been shown (*supra vide*) that no precipitin can be detected in the superfluid at the end of an interaction, provided that the amount of homologous protein exceeds a certain quantity. Table vi. records some of the results obtained by testing the superfluid with a further addition of protein and noting the presence or absence of a further precipitate. In the series to be described, the quantity of protein was sufficient to neutralise or precipitate* the precipitin in the antiserum. The superfluids were considered free from precipitin since they yielded no precipitate on the addition of 144 mg. dried egg-white. The results are recorded in Table ix.

Table ix.

No. of tube.	Weight of dried egg-white in milligrams.	Volume of antiserum in cubic centimetres.	Volume of saline solution in cubic centimetres.	Weight of precipitate in milligrams.	Weight of precipitate in milligrams from 1 c.c. antiserum.
1	14·4	2 c.c.	50 c.c.	3·2	1·6
2	36·0	2 c.c.	50 c.c.	3·5	1·75
3	144·0	2 c.c.	50 c.c.	3·4	1·7
4	432·0	2 c.c.	50 c.c.	3·4	1·7

Here the antiserum was formed by the injection of hen's egg-white, and the fresh antiserum allowed to interact with fresh egg-white. A portion of the egg-white was dried to determine the solid content of the solution. The precipitates are stated in terms of the amount yielded with 1 c.c. antiserum. The weight

* Compare Welsh and Chapman, Proc. Roy. Soc. London, B.78, p.297, 1906.

of precipitate remains practically constant although the protein increases from 14 gm. to 432 gm. A somewhat similar series is recorded in Table x. In this series 3 c.c. antiserum were allowed to interact with 70, 140, 280 and 560 mg. dried egg-white. After 24 hours the superfluids were removed; to the superfluid of the first tube 70 mg. dried egg-white were added, to the second tube 140 mg. dried egg-white were added, and the remaining tubes were tested in the usual way. The precipitates were treated in the manner above described and weighed. No precipitates occurred in the secondary tubes 3 and 4.

Table x.

Tube.	Weight of dried egg-white.	Amount of antiserum.	Amount of saline solution.	Weight of precipitate.	Total weight of the precipitates.	Total amount of precipitate from 1 c.c. antiserum.
1	70 mg.	3 c.c.	50 c.c.	2·8 mg.	3·5 mg.	1·16 mg.
1B	plus 70 mg.			0·7 mg.		
2	140 mg.	3 c.c.	50 c.c.	3·2 mg.	3·2 mg.	1·06 mg.
2B	plus 140 mg.			trace.		
3	280 mg.	3 c.c.	50 c.c.	3·0 mg.	3·0 mg.	1·0 mg.
4	560 mg.	3 c.c.	50 c.c.	3·2 mg.	3·2 mg.	1·06 mg.

The superfluids from tubes 2B and 1B were tested for the presence of precipitin by the addition of more protein, but no precipitates were obtained. In this series the weight of precipitate obtained from 1 c.c. antiserum remains constant despite the large increase in the quantity of protein. No stress can be laid on the amount of precipitate in tube 1B, since it is doubtful whether the interaction in tube 1 was complete in 24 hours.

As it appears that the precipitate from a given quantity of antiserum is constant, provided there be sufficient protein to neutralise the precipitin, a series of experiments in which the amount of antiserum was varied may be considered. With these experiments may be considered one in which a duplicate was carried out. The details of the experiments were varied to avoid errors. The results are recorded in Table xi.

These results show that the amount of precipitate yielded by each antiserum is a fixed quantity for each cubic centimetre of antiserum. It must be noticed that the amount of saline solu-

Table xi.

Tube.	Antiserum.	Amount of antiserum	Weight of dried protein.	Amount of saline solution.	Weight of precipitate.	Weight of precipitate from 1 c.c. antiserum.
1	Horse-serum 57	2·5 c.c.	100 mg.	50 c.c.	3·7 mg.	1·5 mg.
2	,,	2·5 c.c.	100 mg.	,,	3·5 mg.	1·4 mg.
3	Hen egg-white 59	2·0 c.c.	134 mg.	,,	8·6 mg.	4·3 mg.
4	,,	3·0 c.c.	134 mg.	,,	12·5 mg.	4·2 mg.
5	,,	4·0 c.c.	134 mg.	,,	16·7 mg.	4·2 mg.
6	Horse-serum 53	2·5 c.c.	50 mg.	,,	2·0 mg.	0·8 mg.
7	,,	5·0 c.c.	200 mg.	,,	4·0 mg.	0·8 mg.
8	Horse-serum 56	5·0 c.c.	100 mg.	,,	10·4 mg.	2·1 mg.
9	,,	10·0 c.c.	100 mg.	,,	20·0 mg.	2·0 mg.
10	Hen egg-white 64	1·0 c.c.	28 mg.	,,	1·4 mg.	1·4 mg.
11	,,	2·0 c.c.	28 mg.	,,	2·2 mg.	1·35 mg.
11B	,,		plus 140 mg.		0·5 mg.	
12	,,	3·0 c.c.	28 mg.	,,	3·2 mg.	1·4 mg.
12B	,,		plus 56 mg.		1·0 mg.	
13	,,	4·0 c.c.	28 mg.	,,	3·6 mg.	1·5 mg.
13B	,,		plus 56 mg.		2·4 mg.	

tion used as a diluent is the same throughout the series. In tubes 1 and 2, 2·5 c.c. antiserum for horse-serum interacted with 100 mg. dried horse-serum, and the duplicates agree well. In tubes 3, 4, and 5, 2 c.c., 3 c.c., and 4 c.c. fowl's egg-white antiserum interacted with 100 mg. dried egg-white, yielding 8·6 mg., 12·5 mg., and 16·7 mg. precipitate respectively. Calculating the amount for each c.c. antiserum, it is found to be about 4·2 mg. in each case. In tubes 6 and 7, 2·5 c.c. antiserum for horse-serum reacted with 50 mg. horse-serum, and 5·0 c.c. antiserum reacted with 200 mg. horse-serum. The amount of precipitate in the second tube was exactly double that in the first tube. In tubes 8 and 9, 5 c.c. and 10 c.c. antiserum each interacted with 100 mg. dried egg-white, and the weights of the precipitates agree sufficiently well. In tubes 10, 11, 12, and 13, 1 c.c., 2 c.c., 3 c.c., and 4 c.c. antiserum for fowl's egg-white each reacted with 28 mg.

dried egg-white. After 48 hours the superfluids were removed and a quantity of dried egg-white dissolved in saline solution added to each superfluid. No further precipitate formed in tube 1, but precipitates formed in 11B, 12B, and 13B. Here again there is fair agreement in the amount from each 1 c.c. antiserum.

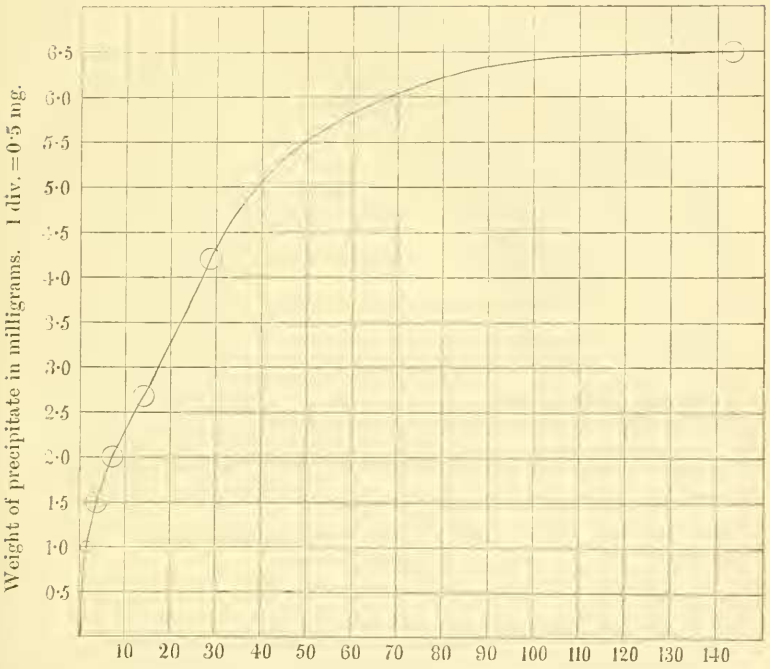
When the amount of protein is insufficient to neutralise all the precipitin in a given quantity of antiserum, the amount of precipitate is diminished. An experiment showing the relation of the precipitate to the amounts of the interacting bodies may be now described. A rabbit was immunised by the injection of 9·6 gm. dried egg-white in eight doses. The antiserum was employed fresh. A solution of egg-white was used for the homologous protein and the solid content determined by drying a fixed quantity. The quantities employed and the results are recorded in Table xii.

Table xii.

Tube.	Amount of antiserum.	Weight of protein.	Amount of saline solution.	Weight of precipitate.	Weight of precipitate from 1 c.c. antiserum.
1	3 c.c.	1·44 mg.	50 c.c.	1·0 mg.	0·33 mg.
2	3 c.c.	3·6 mg.	50 c.c.	1·5 mg.	0·5 mg.
3	3 c.c.	7·2 mg.	50 c.c.	2·0 mg.	0·66 mg.
4	3 c.c.	14·4 mg.	50 c.c.	2·7 mg.	0·9 mg.
5	3 c.c.	28·8 mg.	50 c.c.	4·2 mg.	1·4 mg.
6	3 c.c.	14·4 mg.	50 c.c.	6·5 mg.	2·2 mg.

It will be seen that the amount of antiserum was 3 c.c. in each tube. This quantity yields only a small precipitate in the tubes 1 and 2, so that great stress cannot be placed on these figures. The weights of the precipitates have been set in the graph appended(fig.1). The curve tends to be of a regular form. The amount of antiserum obtained from a rabbit is not usually more than 20 c.c., so that extended series cannot be carried out with antisera from rabbits. It was not considered legitimate to employ mixed antisera. Another series gave a graph of similar form. At present the data are too few to discuss these results at length, in order to determine the type of the interaction.

The effect of the degree of dilution on the weight of precipitate may be now considered. Two series of experiments were carried out. In the first series the quantity of egg-white was constant,



Curve of weight of precipitate formed with increasing weights of homologous protein : 3 cc. antiserum interact with ascending weights of dried egg-white.

and the amount of saline solution used to dilute the interacting masses was varied. In the second series the quantity of egg-white was maintained at a constant concentration in the saline solution. The antisera employed were two fowl's egg-white antisera prepared from rabbits. The results are recorded in Table xiii.

The results of both series correspond, though the absolute amount of precipitate from each antiserum was different. With a quantity of saline solution of 25 c.c., there was a reduction in

Table xiii.

Tube.	Amount of antiserum.	Weight of egg-white.	Amount of saline solution.	Weight of precipitate.	Weight of precipitate from 1 c.c. antiserum.
1	5 c.c. A	100 mg.	25 c.c.	10·8 mg.	2·2 mg.
2	5 c.c. A	100 mg.	50 c.c.	16·2 mg.	3·2 mg.
3	5 c.c. A	100 mg.	100 c.c.	15 mg.	3·0 mg.
4	5 c.c. B	100 mg.	25 c.c.	10·6 mg.	2·1 mg.
5	5 c.c. B	200 mg.	50 c.c.	19·5 mg.	3·9 mg.
6	5 c.c. B	400 mg.	100 c.c.	17·7 mg.	3·6 mg.

the amount of precipitate. All observers have noted this reduction, which has been usually ascribed to a solvent action of the concentrated serum. With a quantity of saline solution of 100 c.c., the precipitates were also reduced slightly. This reduction was probably due to incomplete reaction in 48 hours, since the superfluids removed from tubes 3 and 6 yielded small precipitates on standing for another 48 hours. These results are in accord with the qualitative results previously* obtained.

Determination of Specific Relations.

It has already been shown that, under the conditions before described, a given quantity of antiserum gives rise to a certain weight of precipitate, provided a sufficient amount of homologous protein be present. If the protein of the homologous species be replaced by the protein of a closely related species (as tested by the biological method) the amount of precipitate from that quantity of antiserum is diminished.

It is not practicable to weigh the precipitate from a given quantity of antiserum interacting with a quantity of unknown protein, as a means of differentiation of proteins. This same

* Welsh and Chapman, Journ. of Hygiene, vi., p.256, 1906.

principle adapted to other circumstances has been employed to distinguish between closely related proteins, *e.g.*, those of avian eggs. As an example, an experiment may be considered which records the interaction between an antiserum for fowl's egg-white and the egg-white of the hen, duck, quail, ostrich, partridge, and pheasant, by which the heterologous egg-whites of the different eggs were clearly distinguished from the hen's egg-white. The antiserum was derived from a rabbit which had received six injections of egg-white containing 6·27 gm. dried egg-white. When the rabbit was killed, the antiserum was dried *in vacuo* over calcium chloride at 37°C. At the time the experiments were performed, the dried antiserum was over two months old. For the experiment, 0·13 gm. dried antiserum was dissolved in 5·2 c c. saline solution. The antiserum was thus diluted so that 0·4 c.c. solution was equivalent to 0·01 gm. dried antiserum, 0·2 c.c. solution to 0·005 gm. antiserum, 0·08 c.c. solution to 0·002 gm. antiserum, 0·04 c.c. solution to 0·001 gm. antiserum, and 0·02 c.c. solution to 0·0005 gm. antiserum. To interact with the antiserum, 1 c.c. egg-white of each kind of egg was diluted with 99 c.c. saline solution, and 0·1 c.c. of the solution of each

Table xiv.

Tube.	Weight of antiserum.	Amount of the original solution of antiserum.	Amount of saline solution added to solution of antiserum.	Amount of diluted egg-white, fowl, duck, ostrich, pheasant, partridge, and quail.
1	0·01 gm.	0·4 c.c.	0·1 c.c.	0·1 c.c.
2	0·005 gm.	0·2 c.c.	0·3 c.c.	0·1 c.c.
3	0·002 gm.	0·08 c.c.	0·42 c.c.	0·1 c.c.
4	0·001 gm.	0·04 c.c.	0·46 c.c.	0·1 c·c.
5	0·0005 gm.	0·02 c.c.	0·48 c.c.	0·1 c.c.
6	none.	none.	0·5 c.c.	0·1 c.c.

kind of egg-white placed in each of six tubes. The antiserum was measured out in quantities of six times that required for each tube, and saline solution added in such quantity that the

amount of diluted antiserum for each tube measured 0·5 c.c. In this way it was possible to measure the small amounts of antiserum with some approach to accuracy. The quantities of the interacting bodies are recorded in Table xiv.

Tubes of 4 to 5 mm. bore were employed. Each tube contained 0·6 c.c. fluid. The readings of the precipitates after 48 hours are given in Table xv.

Table xv.

Amount of antiserum	Fowl's egg-white.	Duck's egg-white.	Pheasant's egg-white.	Partridge's egg-white.	Quail's egg-white.	Ostrich's egg-white.
0·01gm.	2·5 mm.	1·0 mm.	1·0 mm.	0·8 mm.	0·8 mm.	0·5 mm.
0·005gm.	1·0 mm.	0·3 mm.	0·5 mm.	0·5 mm.	0·3 mm.	0·3 mm.
0·002gm.	0·3 mm.	trace.	trace.	trace.	trace.	trace.
0·001gm.	trace.	trace.	trace.	none.	none.	trace.
0·0005gm.	trace.	none.	none.	none.	none.	none.
none.	none.	none.	none.	none.	none.	none.

The results show that the amount of precipitate with fowl's egg-white was 2·5 mm., greater than the precipitate with any heterologous protein. It is also evident that, by testing with diminishing quantities of antiserum, the differentiation is easily made. Although 43 tubes were employed, including controls of antiserum and saline solution, and protein and saline solution, the amount of dried antiserum employed was only 0·13 gm., equal to 1·3 c.c. fresh antiserum. The method is therefore economical with material. When the quantity of heterologous protein interacting with 0·01 gm. dried antiserum is increased to produce the maximum precipitate, the amount of precipitate is less than the full precipitate for that amount of antiserum yielded by the homologous protein. This is evident from the results recorded in Table xvi. This table records the results of an experiment similar to that recorded in Tables xiv. and xv., tube 1, but carried out with another antiserum. After 48 hours, the superfluids were removed to clean tubes and treated with a second 0·1 c.c. solution of protein.

The readings show that the combined precipitates with the heterologous protein did not equal the precipitate with the homologous protein.

Table xvi.

Tube.	Weight of antiserum.	Amount of diluted egg-white.	Reading in 48 hours.	Addiment to superfluid.	Reading after 48 hours.
1	0·01 gm.	0·1 c.c.(fowl).	1·5 mm.	0·1 c.c.(fowl).	0·3 mm.
2	0·01 gm.	0·1 c.c.(duck).	0·5 mm.	0·1 c.c.(duck).	none.
3	0·01 gm.	0·1 c.c.(ostrich).	0·5 mm.	0·1 c.c.(ostrich).	0·5 mm.

Another method of differentiation of closely related proteins has been described by Welsh and Chapman.* This method depends on the inhibition of the formation of precipitate by heated antisera, *i.e.*, on the solution of precipitate by heated antisera. It is not, however, so simple as that described above, as it involves a thorough knowledge of the inhibitory powers of the antisera employed, and requires a detailed examination of each antiserum before use. The results obtained in the research on crossed inhibition led Welsh and Chapman to suggest that the precipitate given by hen-egg antiserum and ostrich or any egg albumen other than hen-egg albumen might be regarded as similar to that produced by ostrich-egg antiserum and any egg albumen other than ostrich-egg albumen. It could be assumed that this precipitate resulted from the general avian character or component of the proteins used in immunisation, while the greatly increased precipitate produced by hen-egg albumen and hen-egg antiserum, or by ostrich-egg albumen and ostrich-egg antiserum could be assumed to be due to the specific hen or ostrich character or component of the material used for injection. In this connection, some observations made on the eggs used for the experiment recorded in Tables xiv. and xv., may be noted. After

* The principles of this method are outlined by Welsh and Chapman, Proc. Roy. Soc. London, B.79, p.471, 1907.

48 hours the reactions recorded in Table xv. were complete and the precipitates were read. The superfluids of tubes 1 were removed and to the superfluids certain addiments of solutions of egg-white were made. The solutions were those used in the primary experiments. The observations are recorded in Table xvii.

Table xvii.

Tube.	Original protein present.	Addiment of protein.	Amount of precipitate after 48 hours.
1	0·1 c.c.(fowl).	0·1 c.c.(ostrich).	none.
2	0·1 c.c.(duck).	0·1 c.c.(ostrich).	trace.
3	0·1 c.c.(pheasant).	0·1 c.c.(partridge).	0·5 mm.
4	0·1 c.c.(partridge).	0·1 c.c.(fowl).	1·5 mm.
5	0·1 c.c.(quail).	0·1 c.c.(partridge).	none.
6	0·1 c.c.(ostrich).	0·1 c.c.(duck).	0·5 mm.

In the interpretation of these results, those recorded in Table xvi., must be considered. The presence or absence of a precipitate shows that in tube 1 the additional ostrich egg-white failed to produce a precipitate, as the general avian precipitin was already completely neutralised; in tube 2 there was no precipitate for the same reason; in tube 3 the additional egg-white of the partridge neutralised and precipitated the remaining precipitin; in tube 4 the fowl egg-white neutralised the specific precipitin for the fowl egg-white, yielding therefore a large precipitate; in tube 5 the partridge egg-white failed to reveal any general avian precipitin remaining present; and in tube 6 the duck egg-white neutralised and precipitated the remaining general avian precipitin. These results have also an interest in the light of the similar properties of certain hæmolytic sera. The amboceptors present in these latter, combine with the corpuscles from one species of animal, but after the corpuscles of this species have extracted all the amboceptors capable of union with the corpuscles, the corpuscles of a second species will unite with other amboceptors present in the hæmolytic sera.

Observations on the Precipitins for Vegetable Proteins.

The study of the biological relationships of plants by means of precipitins is much more difficult than those of animals. This is due to the difficulty of obtaining material in which reactions, other than those between the interacting precipitin and protein, do not occur. In the first place, the solution of proteins for intraperitoneal injection of the rabbits must be prepared free from toxic substances. Such solutions must also be sterile. The careful researches* of Dr. J. M. Petrie, in this laboratory, on the seeds of the Acacias, have led to the choice of the proteins of these seeds for preparing the antisera. As further work on the decomposition-products of these proteins is likely to be carried out, and as these proteins may differ widely in chemical properties from the proteins of blood-sera, it may be possible to determine by chemical means whether the precipitates formed in the precipitin reactions are composed of substances akin to the proteins of the Acacias. Recent investigations† of the acidic properties of the proteins and their union with bases, also offer means for the preparation of purer solutions of proteins than have hitherto been obtained. As extracts of the seeds of the Acacias made with 10 % salt solution contain little toxic matter, these extracts have been employed for injection after filtration and dilution. They have been sterilised by heating to 55°C., from 3 to 6 hours. Antisera prepared with heated proteins have been studied by Schmidt.‡ They may be used for the same purposes as antisera prepared with unheated protein. Similar but less extensive experiments with antisera made with heated protein have been carried out in this laboratory with the same results.

The antisera prepared by the injection of these heated proteins have been allowed to interact with solutions of the seeds of different species of plants. The solutions were made by extract-

* Proc. Linn. Soc. New South Wales, 1908, xxxiii., p. 835.

† Brailsford Robertson, "The Proteins," University of California Publications, 1909.

‡ Biochem Zeitschr. xiv., S. 294, 1908.

ing 0·5 gm. powdered seeds with 10 c.c. saline solution. The extracts were filtered and divided into two parts; one part was then heated to 70°C. for 4 hours and filtered, while the second part was unheated. The heated extract was sterile, and the tubes did not show bacterial changes for some days. The unheated extract soon showed signs of putrefaction. In addition to the usual controls, it was necessary to arrange, each time, a series of tubes in which normal rabbit's serum interacted with the extracts of the seeds. The importance of this control is evident from Table xviii.

Table xviii.

Tube.	Seeds.	Amount of extract.	Amount of rabbit's serum.	Precipitate with	
				Unheated extract.	Heated extract.
1	*Acacia pycnantha.*	0·5 c.c.	0·1 c.c.	none.	none.
2	*Sinapis alba.*	,,	,,	0·5 mm.	2·0 mm.
3	*Albium porrum.*	,,	,,	2·0 mm.	2·5 mm.
4	*Chattachlora italica.*	,,	,,	0·5 mm.	3·0 mm.
5	*Casuarina distyla.*	,,	,,	trace.	0·5 mm.
6	*Humea elegans.*	,,	,,	none.	none.
7	*Astroloma pinifolium.*	,,	,,	2·0 mm.	none.
8	*Hemicyclia australasica.*	,,	,,	trace.	none.
9	*Endiandra Sieberi.*	,,	,,	solid.	8·0 mm.
10	*Macadamia ternifolia.*	,,	,,	0·3 mm.	none.
11	*Solanum verbascifolium.*	,,	,,	none.	none.
12	*Podocarpus elata.*	,,	,,	trace.	none.
13	*Pimelea ligustrina.*	,,	,,	none.	trace.
14	*Eucalyptus sideroxylon.*	,,	,,	none.	0·5 mm.
15	*Ficus rubiginosa.*	,,	,,	trace.	1·0 mm.
16	none(control).	,,	,,	none.	—

It is seen that 11 out of the 15 seeds gave extracts which yielded precipitates with normal serum. These extracts could not be used for tests with antiserum.

A series may now be recorded in which the preliminary tests and controls were satisfactory. This experiment is recorded in Table xix.

The antiserum was prepared by the injection of extracts of the seeds of *Acacia pycnantha* containing 3·0 gm. dried protein, given

in six doses. The tests were carried out by mixing 0·1 c.c. antiserum and 0·5 c.c. extract of the seeds to be tested. Both heated and unheated extracts of the seeds were employed. The controls are omitted from the table.

Table xix.

Tube.	Natural Order.	Species.	Heated extract.	Unheated extract.
1	Leguminosæ.	*Acacia pycnantha.*	ppt.	ppt.
2	,,	*A. penninerris.*	ppt.	ppt.
3	,,	*A. neriifolia.*	ppt.	ppt.
4	,,	*A. leptoclada.*	ppt.	ppt.
5	,,	*A. accola.*	ppt.	ppt.
6	,,	*A. spectabilis.*	ppt.	ppt.
7	,,	*A. pendula.*	ppt.	ppt-
8	,,	*Pisum sativum.*	ppt.	ppt.
9	,,	*Phaseolus lunatus.*	nil.	nil.
10	,,	*Vicia faba.*	nil.	—
11	,,	*Medicago sativa.*	nil.	ppt.
12	Gramineæ.	*Triticum vulgare.*	nil.	nil.
13	,,	*Avena sativa.*	nil.	—
14	Myrtaceæ.	*Eucalyptus sideroxylon.*	nil.	nil.
15	Coniferæ.	*Podocarpus elata.*	nil.	nil.
16	Solanaceæ.	*Solanum verbascifolium.*	nil.	nil.
17	Epacrideæ.	*Astroloma pinifolium.*	nil.	nil.
18	Compositæ.	*Humea elegans.*	nil.	nil.
19	Proteaceæ.	*Macadamia ternifolia.*	nil.	—

Quantitative differences in the amounts of the precipitates are not recorded in the table. In the first place, it may be noted that the seeds of the Natural Order of the Leguminosæ are marked off clearly from the seeds of the other well marked Natural Orders. In the second place, certain of the seeds of the Leguminosæ do not react with the antiserum for *Acacia pycnantha.* These results have been repeated with other antisera with similar results. It is probable that the precipitin test will be equally useful in the vegetable kingdom as in the animal kingdom. It possesses also the possibility of supplying data* for a correct appreciation of the value of morphological characters in the differentiation of species.

*An extended research on this aspect is being carried out in collaboration with Dr. Petrie.

The Relation of Deviation of Complement to Precipitum.

Moreschi, in his first paper* on the phenomena of deviation, showed that the fixation of complement appeared only as a sequel of precipitation. In his second paper† he was less decided on this point, but held that the amount of complement fixed was proportional to the amount of precipitate. Neisser and Sachs‡ were of opinion that the essential feature in the deviation of complement was the union of a substance (antigen) with its antibody. Gay§ regarded the precipitate as the all-important factor in the fixation of complement. He found that, after the precipitate had formed, the superfluid did not bind complement, but that the precipitate, even after thorough washing, fixed complement. Muir and Martin‖ investigated the relation of deviation of complement to the precipitin test. They concluded, *inter alia*, that a mixture of serum and its antiserum had the property of fixing or deviating complement and thus interfering with hæmolysis, that the amount of homologous protein necessary to produce a distinct deviation of complement was extremely small, 0·00001 c.c. or even less—as a rule it was many times less than the amount necessary to give a visible precipitate with the antiserum—that when a precipitate formed, the deviating substance was present in the precipitate and might be so exclusively, and that the precipitation was not essential to the deviation-phenomena, as these could be given without the formation of a precipitate. Welsh and Chapman¶ have, however, found consistently that precipitates form in 48 hours with quantities as small as those employed by Muir and Martin. Stress need not be laid on this point, as there is now general agreement that it is the product of the interaction of antigen and antibody, and not a

* Berl. klin. Wochenschr. S.1181, 1905.
† Berl. klin. Wochenschr. S.76, 1906.
‡ Berl. klin. Wochenschr. S.1388, 1905.
§ Ann. de l'Inst. Pasteur, xix., p.593, 1905.
‖ Journ. of Hygiene, vi., p.265, 1906.
¶ Journ. of Hygiene, vi., p.259, 1906.

simple mixture of these substances, that has to do with fixation of complement.

Moll,* Welsh and Chapman,† and Rodet‡ have brought forward much evidence to show that the precipitate or final product of the interaction of antiserum and homologous protein is mainly derived from the antiserum. Michaelis,§ in his recent summary of precipitins, does not accept the general conclusion, but grants that with the quantities employed by Welsh and Chapman this conclusion holds. He writes "that these observers have only had regard to mixtures of much precipitin and little precipitable substance . . . . . and have rightly concluded that the precipitate consists wholly or almost wholly of the proteins (Eiweisskörpern) of the precipitin serum." Now the quantities of which Michaelis is writing show a much greater proportion of homologous protein to antiserum than those employed by Muir and Martin and other workers on deviation of complement. Disregarding for the present purpose the source of the precipitate under all circumstances, it can be accepted that the precipitate which usually brings about deviation of complement is derived mainly from the antiserum. It therefore follows that the amount of complement deviated will be proportional to the amount of antiserum used as antibody. It has been shown above that an increase in the amount of antiserum leads to an increase in the amount of precipitate, so that by increasing the amount of antiserum (antibody) deviation of complement may be obtained with smaller amounts of homologous protein. This factor has not yet received attention in work upon deviation of complement.

Remarks upon the Practical Applications of the Precipitins.

As pointed out in the introduction, the precipitin test was applied early to the diagnosis of the source of blood-stains.

* Quoted by Rodet, *loc. cit.*

† Proc. Roy. Soc. London, B.lxxviii., p.310, 1906.

‡ Comp. rend. Soc. Biol. Paris, p.671, 1906.

§ Oppenheimer, Handbuch der Biochemie, Jena, 1909, Bd.ii., Hft.1, S.565.

During the subsequent years the range of application has been much extended.* The increased attention to public health and food-supply has led to the use of the test in analytical work concerned with meat and animal foods generally. A detailed account of the methods employed in the use of the test in routine examinations in a municipal laboratory is given by Fornet and Müller.† The test has also been utilised in medical diagnosis. The application of the test to the diagnosis of hydatid disease, so prevalent in this country, may be cited. The small amount of manipulation required for these tests, and the speed with which a result may be obtained, give this test a great advantage over the test for deviation of complement which is used for the same purposes.

* For a summary of the practical applications of the test, see Welsh and Chapman, Aust. Med. Gaz., 1907, p.1.

† Zeit. f. biol. Meth. und Tech. i., p.201, 1908.